COMPOSITION OF FUNGUS HYPHAE

III. THE PYTHIACEAE[1]

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The genus *Pythium*, established by Pringsheim in 1858, has long been recognized as an interesting group of fungi (1). The economic importance of this genus was first indicated by Hesse in 1874. Since that time appreciation of the importance of this group has increased as agricultural production has become more intensified.

Interest in the Pythiaceae, aside from their economic importance as damping-off fungi, has been chiefly from the standpoint of morphology and cytology. Very little is known regarding the nature of the hyphae or the by-products of metabolic activity. This report is concerned with the composition of the mycelium. It has been the purpose of this investigation to determine the nature of the substances occurring in the mycelium, to identify them, and to learn how they are arranged.

SPECIES INVESTIGATED

The type species, viz., *P. monospermum* and *P. entophylum*, used by Pringsheim in establishing the genus *Pythium* could not be obtained. In this study, six identified species of Pythium obtained from the American Type Culture Collection, and three unidentified isolates were investigated. The group of known species included *P. debaryanum* Hesse, *P. vexans* deBary, *P. ultimum* Trow, *P. acanthicum* Drechsl., *P. aphanidermatum* (Eds.) Fitz., *P. spinosum* Sawada, and three species isolated, respectively, from diseased tomato seedlings, begonia, and potato.

PLAN OF STUDY

Cultures were developed in 300-cc. flasks containing 125 cc. of potato broth filtered free of all sediment. This medium was found to be very satisfactory as well as easy to prepare in quantity. Richard's solution with saccharose as the carbohydrate permitted only meager growth. With glucose substituted for saccharose, development was much better, and the addition of 0.1 per cent of yeast extract still further improved the quality of the medium for *Pythium*. Although the modified synthetic solution was used in the first part of the work, it was soon replaced with potato broth, which was more satisfactory.

Investigation of the mycelium of each species was made at different periods of growth, varying from 3 days to 3 months. For the most part, only fresh cultures were used. When it was not possible to complete the analysis at a definite stage of development in the time available, the mats were washed free of culture solution and preserved in 95 per cent alcohol until required for use. This was found to be particularly important with *Pythium*, because a growth in a favorable medium, the first few days, is rapid.

Both microchemical and macrochemical methods were employed. Only small quantities of hyphae were used in many of the tests: the amounts were, in most cases, equivalent to less than 0.05 gm. of dried mycelium. The general procedure consisted in the detection, removal, and identification of the different constituents of which the hypha is made up. Appropriate physical and chemical tests and solvents were used. The technique was based upon procedure described by Molisch (4), Tunmann (8), Klein (2), Morrow (5) and Campbell (9).

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Examination of young vigorously growing hyphae 2 to 3 days old showed that the material on the outside is not anisotropic or doubly refractive. This property gradually changed. After 5 to 7 days of growth, at 25 degrees C., the older portions of the hyphae become dimly anisotropic; and as the culture advanced in age, this physical property became more evident, yet the young, newly formed, hyphal tips were still found to be dark when viewed in polarized light. Old cultures that had ceased activity after 3 months of growth were anisotropic throughout the entire extent of the mycelial filaments.

When very young hyphae were immersed in a weak solution of ruthenium oxychloride, the dye became fixed in the walls and could not be washed out with water. This is a presumptive test for pectic compounds. For futher confirmation of the presence of pectic material, several mats, washed free from culture liquid, were then placed in 0.5 per cent aqueous solution of NaOH and allowed to stand for several hours with occasional shaking. The alkali not only dissolved the pectic substance from the hyphae, but also changed it to pectin. The liquid was filtered and divided into two portions. One portion was rendered acid with HCl, and to the other was added an excess of $CaCl_2$. A gelatinous precipitate occurred in both instances. The first was pectic acid, the second, calcium pectate.

The precipitates were collected by centrifuging and portions tested with the orcinol-HCl reactions. A color change occurred ranging from pink to violet and blue or blue-green. When a trace of $FeCl_3$ was added after the pink color had developed, the transition to green took place promptly. In all cases the color could be removed by shaking with amylic alcohol.

After removal of the pectic material, the hyphae were found to be doubly refractive even in young cultures. Other cell wall substances of fungi which are known to possess this property are callose, and cellulose. Chitin of lobster carapace is anisotropic en masse, but when it occurs in fibers or strands this characteristic is less conspicuous.

Very young cultures which had been rendered free of pectic compounds by aqueous alkali, and old cultures in which the pectic material had been decomposed by autolysis did not fix dyes. This was anticipated, because previous investigations of the *Fusaria* and *Sclerotinia* groups by the author (6, 7) had demonstrated the lack of affinity of the hyphae of those groups for stains. This lack was found to be due to the inert character of the fatty acids with which the mycelium was impregnated. When the hyphae at this stage of the analysis were treated with 1 per cent alcoholic potash by refluxing, the liquid at first became yellow in color, deepening in intensity until a maximum was reached. After the hyphae had been washed in alcohol until all alkali was removed, treatment with 1 per cent aqueous potash did not separate any material. Callose would be soluble in aqueous potash after the fatty acids had been saponified and dissolved from the hyphae (7). Treatment with bromine water for 24 hours followed by 1 per cent aqueous alkali also failed to render any cell wall substance soluble.

With the probability of the presence of callose eliminated, attention was now directed to the detection and identification of cellulose, since the hyphae were more strongly doubly refractive after the removal of fatty acids and fixed such aniline dyes as methylene blue, congo red, and safranin.

A test for cellulose was next made. Lugol's iodine solution followed by 70 per cent sulfuric acid caused the mycelium to develop a blue color, which was indicative of cellulose. Old, partially autolyzed cultures demonstrated a blue color with the same test, yet much more quickly after previous treatment with alcoholic potash to remove fatty acids. Young cultures, 3 to 10 days old, without any other treatment, showed only the yellow or brown color of the iodine, and no blue absorption compound characteristic of cellulose developed with the addition of sulfuric acid.

Cellulose is soluble in ammoniacal cupric hydrate. This reagent had no solvent action upon young hyphae less than a week old; yet, when mycelium of the same age, with pectic material and fatty acids removed, was subjected to the reagent, cellulose passed into solution. This was demonstrated by filtering off the liquid through a glass filter, diluting the filtrate with several volumes of water, and acidifying with acetic or hydrochloric acid. With this treatment, cellulose was precipitated. This precipitate was doubly refractive in polarized light, gave a blue color with iodine and sulfuric acid, and upon hydrolysis, yielded a reducing sugar from which a phenylosazone was prepared which was found to be identical in appearance and melting point with the dextrosozone.

The same result was accomplished by hydrolyzing the cellulose with 70 per cent sulfuric acid. Both mycelium and acid were cooled in a refrigerator to 8 degrees C. before the mycelium was placed in the acid. After 12 hours sufficient water was added to reduce the strength of the acid to 3.5 per cent, and hydrolysis was completed by refluxing.

The solid residue in both cases, washed free of the reagents, no longer gave a test for cellulose, was no longer doubly refractive in polarized light, and did not fix aniline dyes.

This residue was tested for chitin. It was boiled for 30 minutes in saturated KOH solution, then washed and hardened in 90 per cent alcohol. A red violet color developed when the substance was placed in Lugol's iodine solution diluted with 2 parts of water. If too strong an iodine solution is used, the color change may take place so rapidly that the violet color may appear black. In addition to the chitosan reaction, chitosan sphaerites and glucosamine hydrochloride were prepared. Thus, the residual portion of the *Pythium* complex was identified as chitin.

SUMMARY

An analysis has been made of the mycelium of six different species of *Pythium*, and the same composition has been revealed in all the species investigated.

The outer covering of young hyphae was found to be pectic material. This covering could be removed by suitable solvents to disclose underneath a layer of cellulose. The pectic substance on the outside of young hyphae completely masked the cellulose and prevented reagents from reaching it. Old, mature hyphae are doubly refractive to polarized light and, therefore, devoid of pectic substance on the outside.

The cellulose was strongly impregnated with fatty acids which prevented the fixation of dyes. The fatty acids could be removed by saponifying with alcoholic potash.

The residual or basic skeleton of the Pythium mycelium was identified as chitin.

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